Autoxidation of Biological Molecules. 2. The Autoxidation of a Model Membrane. A Comparison of the Autoxidation of Egg Lecithin Phosphatidylcholine in Water and in Chlorobenzene[1]

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Abstract: The kinetics of the autoxidation of egg lecithin phosphatidylcholine in homogeneous solution in chlorobenzene and as a bilayer dispersion in 0.1 M aqueous NaCl has been studied at 30 °C under 760 torr of O_2. The autoxidations were initiated by the thermal decomposition of di-*tert*-butyl hyponitrite. The efficiency of chain initiation, *e*, was determined by the induction period method using α-tocopherol as the chain-breaking antioxidant. In chlorobenzene *e* was ca. 0.66 but in the aqueous dispersion *e* was only ca. 0.091. The reduced efficiency of initiation in the bilayer is attributed to a reduction in the fraction of *tert*-butoxyls which escape from the solvent cage, and this in turn is due to the fact that the bilayer has a high microviscosity. The rate of autoxidation of the egg lecithin in chlorobenzene is proportional to the lecithin concentration and to the square root of the rate of chain initiation, and is virtually independent of the oxygen pressure, which means that this autoxidation follows the usual kinetic rate law. In the aqueous dispersion the concentration of egg lecithin in the bilayer cannot be altered, but since the rate of autoxidation is proportional to the square root of the rate of chain initiation and is virtually independent of the oxygen pressure, the usual kinetic rate law would also appear to be followed. The oxidizability of egg lecithin in chlorobenzene is 0.61 $M^{-1/2} s^{-1/2}$, and in the aqueous dispension it is 0.0165 $M^{-1/2} s^{-1/2}$. The reduction in oxidizability in the bilayer is attributed to the diffusion of the peroxyl radical center, which is a polar moiety, out of the autoxidizable, nonpolar, interior region of the bilayer and into the nonautoxidizable, polar surface region. As a consequence, chain progogation will be retarded and chain termination will be accelerated.

The autoxidation of most organic substrates in homogeneous solution is a free-radical chain process which, at oxygen partial pressures above ca. 100 torr, can generally be represented by the raction sequence given in eq 1-4.[3-11] In this scheme RH rep-

initiation: formation of R· (or ROO·) rate = R_i (1)

propagation: $R\cdot + O_2 \rightarrow ROO\cdot$ (2)

$$ROO\cdot + RH \xrightarrow{k_p} ROOH + R\cdot \quad (3)$$

termination: $ROO\cdot + ROO\cdot \xrightarrow{2k_t}$ nonradical organic products + O_2 (4)

resents the organic substrate, R· the carbon-centered radical it yields upon reaction with the peroxyl ROO·, and R_i the rate of chain initiation; i.e., the rate at which new chains are started in the system. The rate of oxygen absorption for a system which follows these four reactions can be represented by eq 5, where k_p

$$\frac{-d[O_2]}{dt} = \frac{k_p[RH]R_i^{1/2}}{(2k_t)^{1/2}} \quad (5)$$

and $2k_t$ are the rate constants for the rate-controlling chain propagation step and for the chain termination step, respectively. The rate constant ratio, $k_p/(2k_t)^{1/2}$ is generally referred to as the "oxidizability" of the substrate.

For *quantitative* kinetic studies of autoxidation the rate of chain initiation must be known and controlled. It is generally controlled by using a thermal initiator, In, and working under conditions where new chains are started only by the decomposition of In, eq 6.

$$In \xrightarrow{k_i} 2R\cdot \quad (6)$$

$$\text{e.g., } RN{=}NR \rightarrow 2R\cdot + N_2 \quad (6a)$$

Although the rate constant, k_i, for decomposition of the initiator can usually be determined without difficulty, it will rarely yield the rate of chain initiation. This is because not all the radicals formed in reaction 6 succeed in escaping from the solvent cage in which they are produced, and only that fraction, *e*, which do escape are able to initiate autoxidation chains. The rate of initiation is, therefore, generally determined by the inhibitor method developed by Hammond and co-workers.[12] A known concentration of a chain-breaking phenolic antioxidant, ArOH, which has been shown to react with exactly two peroxyls, i.e., which terminates exactly two oxidation chains, is added to the system, and the length of time, τ, is measured during which oxidation is suppressed. The rate of chain initiation is related to this induction period by the eq 7. The efficiency of chain initiation is given

$$R_i = 2[ArOH]/\tau \quad (7)$$

by eq 8 and the chain length, ν, by eq 9.

$$e = R_i/2k_i[In] \quad (8)$$

$$\nu = (d[O_2]/dt)/R_i \quad (9)$$

The careful application of the simple ideas outlined above has yielded an enormous body of quantitative kinetic information

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(2) N.R.C.C. Visiting Research Officer 1979-1980, on sabbatical leave from Mount Allison University, Sackville, New Brunswick.
(3) "Autoxidation and Antioxidants"; Lundberg, W. O., Ed.; Interscience: New York, 1961; Vols. I and II.
(4) Scott, G. "Atmospheric Oxidation and Antioxidants"; Elsevier: Amsterdam, 1965.
(5) "Oxidation of Organic Compounds", *Adv. Chem. Ser.* **1968**, *Nos. 75 and 76*.
(6) Mayo, F. R. *Acc. Chem. Res.* **1968**, *1*, 193-201.
(7) Ingold, K. U. *Acc. Chem. Res.* **1969**, *2*, 1-9.
(8) Reich, L.; Stivala, S. S. "Autoxidation of Hydrocarbons and Polyolefins"; Marcel Dekker: New York, 1969.
(9) Betts, J. *Q. Rev., Chem. Soc.* **1971**, *25*, 265-288.
(10) Howard, J. A. *Adv. Free-Radical Chem.* **1972**, *4*, 49-173.
(11) Howard, J. A. In "Free Radicals"; Kochi, J. K,., Ed.; Wiley: New York, 1973; Vol. II, Chapter 12, pp 3-62.
(12) Boozer, C. E.; Hammond, G. S.; Hamilton, C. E.; Sen, J. N. *J. Am. Chem. Soc.* **1955**, *77*, 3233-3237.

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regarding the autoxidation of a wide variety of organic substrates in homogeneous solution.[3-11]

Autoxidation in biological systems is, in contrast, totally lacking in *quantitative* kinetic data. This is surprising since it is well-known that polyunsaturated fatty acid such as linoleic and arachidonic acids, which are present as phosphoglyceride esters in lipid membranes, are particularly susceptible to autoxidation. Moreover, autoxidation in biological systems has been associated with such important pathological events as damage to cellular membranes in the process of aging and the action of certain toxic substances.[13-15] Nevertheless, the three following simple, but extremely important, questions regarding the autoxidation of biological membranes have rarely been asked and have never been satisfactorily answered. (1) *How does the efficiency of chain generation by a thermal initiator buried within a biomembrane dispersed in an aqueous environment compare with the efficiency of the same initiator in a homogenous system containing the same autoxidizable organic substrate*? (2) *Does the autoxidation of a biomembrane in an aqueous environment follow the same kinetic rate law as that for organic substrates in homogeneous solution (i.e., eq 5)*? (3) *If the answer to question 2 is yes, how does the oxidizability of the biomembrane in the aqueous environment compare with the oxidizability of the same material in a homogeneous system*?

To answer these fundamental questions on molecular biology, we have selected as a substrate the phosphatidylcholine from egg lecithin since this phospholipid is known to form bilayer dispersions in water which serve as *models* for biological membranes.[16] The properties of these and related vesicles have received considerable attention.[16-18] We hope our answers to the above questions will prove relevent to the autoxidation of biomembranes.

Egg lecithin is known to autoxidize readily, and, in fact, Schrieber[19] has recently reported on its autoxidation in water and in organic solvents. Unfortunately, he neither knew nor controlled the rate of chain initiation and so his results do not provide answers to any of the questions posed above.

Two more recent studies,[20,21] which came to our attention after the present work was complete, do provide partial answeres to some of the above questions though the conclusions reached in both studies differ substantially from our own. Winterle and Mill[20] have concluded that the efficiency of radical production by the thermolysis of azobis[(2-*n*-butylcarboxy)propane] (ABCP) at 50 °C is about half as large in a lipid bilayer as in homogeneous solutions and that the ABCP is located "in a region of moderate-to-low polarity". We find, admittedly with a different initiator, that in a lipid bilayer e has less than one-tenth of its value in homogeneous solution. Second, Porter et al.[21] have analyzed the hydroperoxides formed during the autoxidation of linoleic acid in homogeneous solution and of its phospholipid esters in aqueous emulsion by means of HPLC; a technique which has also been used to analzye the hydroperoxides formed from unsaturated lecithins.[22,23] The authors of this outstanding paper conclude that there is no major difference in the value of k_p/k_{-2}, the ratio of the rate constants for H-atom abstraction to peroxyl radical β-scission (i.e., the reverse of reaction 2), in their two systems.[21] *If* the magnitude of k_{-2} is independent of the environment surrounding the peroxyl radicals, then Porter et al.'s results imply that k_p is the same in a homogeneous solution as in a bilayer dispersed in an aqueous medium. In contrast, our own results suggest that oxidizability is *substantially* less in the aqueous bilayer and that k_p is also probably significantly smaller in the bilayer than in homogeneous solution.

Experimental Section

Materials. Egg Lecithin. Our work began with a sample kindly prepared for us by Anne Joyce (N.R.C.C.). After the initial studies, work continued with a sample purchased from Lipid Products (South Nutfield, nr. Redhill, Surrey, England) as a solution in chloroform–methanol. These solvents were removed under high vacuum, and known concentrations of egg lecithin were stored either in chlorobenzene, for autoxidation in this solvent, or in methylene chloride, for autoxidation in water. These solutions were stored under nitrogen at −25 °C in the dark. The two samples of egg lecithin gave results which were identical within experimental error.

Initiators. The following thermal initiators were employed: di-*tert*-butyl hyponitrite (DBHN);[24] 2,2'-azobis(isobutyronitrile) (AIBN, Eastman, recrystallized from cold methanol); azocumene, and 2,2,3,3-tetraphenylbutane (supplied by J. A. Howard, N.R.C.C.). These initiators were stored as solutions of known concentration in benzene or chlorobenzene at −25 °C in the dark. All were effective initiators in chlorobenzene, but only DBHN was really effective in the aqueous system (vide infra).

Inhibitor. Commercial *d,l*-α-tocopherol (I.C.N. Pharmaceuticals, Inc.) was stored as solutions of known concentration in chlorobenzene or methylene chloride at −25 °C in the dark. In model experiments with Tetralin and AIBN it produced the same induction period as an equal concentration of α-tocopherol which had been purified by HPLC and as an equal concentration of 2,6-di-*tert*-butyl-4-methoxyphenol.

Tetralin. This compound, which was used in calibration runs, was distilled and passed through alumina immediately prior to use.

Solvents. Chlorobenzene was washed with concentrated sulfuric acid, 2% sodium bicarbonate, and water, dried over anhydrous sodium sulfate, and distilled from barium oxide. Methylene chloride and benzene were spectral grade and were used without purification. The water used for the aqueous dispersions of egg lecithin was doubly distilled in all-glass equipment. To this water was added 0.1 M of ultrapure sodium chloride (Ventron).

Autoxidation Procedure. Autoxidations were carried out at 30 °C since this is the temperature which has been used most frequently to obtain quantitative kinetic data on hydrocarbon autoxidations.[10,11] The oxidations were conducted under 760 torr of O_2 (or occasionally air) in an automatic recording gas absorption apparatus similar to that previously described,[25,26] but with a more sensitive pressure transducer (Validyne International P 10-824). For reduction of base lline drift, the apparatus was first "conditioned" by passing a slow stream of oxygen saturated with the appropriate solvent through the equipment. The apparatus was calibrated by monitoring the AIBN-initiated autoxidation of Tetralin under conditions where the rate was also reliably determined by Dr. J. A. Howard (N.R.C.C.). After appropriate conditioning the detailed procedure depended on whether the reaction was run in chlorobenzene or water, as summarized below.

Chlorobenzene. The reaction vessel (volume ca. 4 mL) was charged with 2 mL of chlorobenzene containing a known amount of egg lecithin. The vessel was filled with oxygen, attached to the pressure transducer, and shaken continuously while thermal equilibrium was established. Thereafter, an appropriate amount of initiator in chlorobenzene was injected from a microsyringe through a small stopcock directly into the reactant solution. Appropriate stopcocks were then closed, and the strip chart recorder was started. After the absorption of ca. 1.5×10^{-6} mol of O_2 (corresponding to ca. 1 V on the recorder), the value of R_i was determined by injecting a solution of α-tocopherol through the small stopcock and into the reactant solution and measuring the induction period.[26]

Water. The reaction vessel was charged with egg lecithin in methylene chloride and initiator in benzene. Most of these solvents were removed with a stream of nitrogen in the cold. The remaining traces of solvent were removed by evacuation for 1 min on a water aspirator followed by 2–3 min on a high vacuum line (≤0.1 torr). Prolonged evacuation led to some loss of DBHN, which sublimes quite readily.[24] (See Note Added in Proof.) The value of R_i was determined in an experiment separate from that used to measure the rate of oxidation. The vessel was charged with egg lecithin, initiator, and α-tocopherol, and the solvents were removed as before. Induction periods were measured from the time of

(13) de Duve, C., Hayaishi, O. Eds.; "Tocopherol, Oxygen and Biomembranes"; Elsevier/North-Holland: Amsterdam, 1978.
(14) Mead, J. F. In "Free Radicals in Biology"; Pryor, W. A., Ed.; Academic, New York, 1976; Vol. I, Chapter 2, pp 51–68.
(15) Wolman, W. *Isr. J. Med. Sci.* **1975**, *11*, pp 1–229.
(16) Papahadjopoulus, D.; Kimelberg, H. K. *Prog. Surf. Sci.* **1973**, *4*, Part 2, 141–232.
(17) Popahadjopoulos, D., Ed. *Ann. New York Acad. Sci.* **1978**, *308*, 2–48.
(18) Fendler, J. H. *Acc. Chem. Res.* **1980**, *13*, 7–13.
(19) Schreiber, J. *Pharmazie* **1979**, *34*, 36–40.
(20) Winterle, J. S.; Mill, T. *J. Am. Chem. Soc.* **1980**, *102*, 6336–6338.
(21) Porter, N. A.; Weber, B. A.; Weenen, H.; Khan, J. A. *J. Am. Chem. Soc.* **1980**, *102*, 5597–5601.
(22) Crawford, C. G.; Plattner, R. D.; Sessa, D. J.; Rackis, J. J. *Lipids* **1980**, *15*, 91–94.
(23) Porter, N. A.; Wolf, R. A.; Weenen, H. *Lipids* **1980**, *15*, 163–167.
(24) Kiefer, H.; Traylor, T. G. *Tetrahedron Lett.* **1966**, 6163–6167.
(25) Howard, J. A.; Ingold, K. U. *Can. J. Chem.* **1969**, *47*, 3809–3815.
(26) Burton, G. W.; Ingold, K. U., preceding paper in this issue.

Table I. Autoxidation of Neat (7.34 M) Tetralin at 30 °C under 760 torr of O_2

run no.	$10^4[In]$/ M	$10^9(2k_i[In])$/ $M\ s^{-1}$	10^9R_i/ $M\ s^{-1}$	e	$-10^7d[O_2]/dt$/M s^{-1} measd	corrd[a]	ν[b]	$-10^3d[O_2]/dt$/ $[RH]R_i^{1/2}$[b]/ $M^{-1/2}\ s^{-1/2}$
				[DBHN][c]				
34	7.24	4.64	2.43	0.52	9.11	9.13	376	2.56
36	7.24	4.64	2.64	0.57	10.5	10.5	398	2.83
37	7.35	4.71	2.89	0.61	9.89	9.91	343	2.56
				[AIBN][d]				
27	403	7.50	2.58	0.34	9.60	9.61	373	2.71
39	95.2	1.77	0.655	0.37	5.47	5.48	837	3.06
42	400	7.44	2.50	0.34	10.5	10.6	424	2.89
				[AC][e]				
40	7.39	2.88	1.70	0.59	9.10	9.11	536	3.06
41	7.39	2.88	1.79	0.62	7.91	7.92	443	2.59

[a] Corrected for nitrogen evolution by these initiators ($k_i[In]$) and oxygen absorbed by the initiating radicals ($2ek_i[In]$) and oxygen evolved in chain termination ($ek_i[In]$), i.e., corrected by adding $(1-e)k_i[In]$ to the measured rate. [b] Calcuated from corrected rate. [c] $k_i = 3.2 \times 10^{-6}\ s^{-1}$ (ref 24). [d] $k_i = 9.3 \times 10^{-8}\ s^{-1}$ (ref 28). [e] $k_i = 2.0 \times 10^{-6}\ s^{-1}$ (ref 29).

Table II. Rate Constants for the Thermal Decomposition of DBHN at 30 °C

medium	[egg lecithin]	[DBHN]/M	10^6k_i, s^{-1}
chlorobenzene	25×10^{-3} M[a,b]	3.39×10^{-2} [b]	3.79
chlorobenzene	25×10^{-3} M[a,b]	3.45×10^{-2} [b]	3.77
Nujol		3.50×10^{-2} [b]	4.88
Nujol		2.01×10^{-2} [b]	5.83
0.1 M aqueous NaCl	12.5×10^{-5} mol[a]	23.8×10^{-2} [c]	2.56[d,e]
0.1 M aqueous NaCl	10.0×10^{-5} mol[a]	23.6×10^{-2} [c]	4.13[f]
0.1 M aqueous NaCl	7.5×10^{-5} mol[a]	21.6×10^{-2} [c]	2.53[f]
0.1 M aqueous NaCl	6.25×10^{-5} mol[a]	45.9×10^{-2} [c]	2.26[e]

[a] Based on an assumed average molecular weight of 800 (see text). [b] Units are mol/L of chlorobenzene (or Nujol). [c] Units are mol/L of egg lecithin, it being assumed that the density oi egg lecithin is 0.8 (see text). [d] After 5 half-lives the yield of N_2 evolved was 95% on the basis of the initial quantity of DBHN. [e] Determined by N_2 evolution. [f] Determined by HPLC analysis for DBHN.

insertion into the bath and charging with oxygen to the "break" when autoxidation commenced. In both types of run the aqueous dispersions were prepared by vortex stirring the residual organic material obtained after evaporation of the solvent with 2 mL of 0.1 M aqueous sodium chloride for 5 min under nitrogen. This gave a milky emulsion. Certain dispersions were also sonicated in a bath-type ultrasonc cleaner (Brasonic 220) at 15 °C under nitrogen.

Decomposition of DBHN. The rate of decomposition of the initiator (DBHN) in chlorobenzene containing egg lecithin and in aqueous dispersions of egg lecithin was measured by monitoring the nitrogen evolution in the same apparatus used for autoxidation. The solutions were prepared in the same way as in the autoxidation procedure, and the decomposition was followed under an atmosphere of nitrogen.

The rate of decompsition of DBHN in the egg lecithin dispersions was also measured by an HPLC technique. Aliquots were removed after known time intervals and were diluted with methanol containing toluene as an internal standard. The DBHN concentration was measured directly by HPLC by using a Spectraphysics 8000 HPLC and RP-8 reverse-phase column eluted with 60:40 methanol–water.

Analytical Procedures. The sizes of the vesicles present in the aqueous dispersions were kindly measured by Dr. B. F. Johnson (N.R.C.C.) on a phase contrast microscope (Reichert, No. 258, 427) equipped with a camera and using oil immersion and a 1200X magnification.

The fatty acids present in the egg lecithin before and after autoxidation were kindly analyzed for us as their methyl esters by Anne Joyce (N.R.C.C.). The sample was refluxed 2 h with 4 mL of 0.7 M methanolic HCl. The solution was cooled, ca. 0.5 mL of H_2O was added, and this mixture was then extracted with hexane. Most of the hexane was removed under nitrogen and the residue analyzed by VPC by using a 6 ft × $1^1/_8$ in. glass column of 15% diethylene–glycolsuccinate on chromosorb "W" at 175 °C.

The absolute yield of the main organic products formed by the thermal decomposition of DBHN, viz., di-*tert*-butyl peroxide, *tert*-butyl alcohol, and acetone, was determined by D. Lindsay (N.R.C.C.). Egg lecithin samples containing DBHN in chlorobenzene or 0.1 M aqueous sodium chloride were shaken under O_2 in a sealed vessel at 30 °C for ca. 10 half-lives of the initiator (ca. 25 days). For the chlorobenzene run, the three products of interest were analyzed directly by VPC by using a 24 ft × $^1/_8$ in. column of 20% carbowax 20 M on chromosorb "W" (HP) at 100 °C. However, for the aqueous run most of the di-*tert*-butyl peroxide was found to be in the vapor phase. This created an analytical problem which was overcome by taking the sealed reaction vessel after reaction and sealing it within a second vessel containing 10 mL chlorobenzene and a magnetically operated steel plunger. When the reaction vessel was broken, a two-phase system was obtained, the peroxide being absorbed by the chlorobenzene and the alcohol and acetone being partitioned between the two phases. Analysis by VPC of both phases gave the total yield of all three products.

The solubility of DBHN in water and its partitioning between water and egg lecithin were measured via its absorption at 227 nm ($\epsilon = 62000$) by using a Varian Cary 219 spectrophotometer. The same technique was used to monitor its decay in a methanol–water mixture under sonication. In the partitioning experiment the aqueous phase was separated from egg lecithin vesicles by ultrafiltration[27] using Centriflo membrane cones (CF-50) supplied by Amicon Corp.

Results

The apparatus was calibrated by the autoxidation of neat Tetralin by using di-*tert*-butyl hyponitrite (DBHN), azobis(isobutyronitrile) (AIBN), and azocumene (AC), as the initiators. The kinetic data are summarized in Table I. The rate of chain initiation, R_i, in these and in all other experiments was calculated from the induction periods produced by known concentrations of α-tocopherol.[12,26] This has been shown to be a highly efficient chain-breaking antioxidant which traps two peroxyl radicals.[26] Furthermore, as nature's chosen antioxidant it has an overall molecular structure which should be particularly suited to inhibiting the autoxidation of lipid bilayers. The computed efficiencies of chain initiation by DBHN and AC are ca. 60%, but the efficiency of AIBN is only ca. 35%. The corrections to the measured rates of oxidation (see footnote *a*) are negligible in these experiments because of the relatively high initiation efficiencies and the long chain lengths. The final column serves to confirm eq 5 and gives the oxidizability, i.e., $k_p/(2k_i)^{1/2}$, for Tetralin. The mean value is $(2.78 \pm 0.20) \times 10^{-3}\ M^{-1/2}\ s^{-1/2}$ which is in satisfactory agreement with a literature value of $2.2 \times 10^{-3}\ M^{-1/2}\ s^{-1/2}$ at 30 °C.[30]

The rate constants, k_i, for the thermal decomposition of DBHN were measured in homogeneous solution (chlorobenzene and Nujol) and in aqueous dispersions and are given in Table II. Our results serve to confirm previous work with this initiator which indicates that its decomposition rate does not vary inversely with the viscosity of the medium at atmospheric pressure but is somewhat solvent dependent.[24,31,32] Our measurements in the

(27) Miller, K. W.; Yu, S.-C. T. *Br. J. Pharmacol.* **1977**, *61*, 57–63.
(28) Howard, J. A.; Ingold, K. U. *Can. J. Chem.* **1962**, *40*, 1851–1864.
(29) Nelsen, S. F.; Bartlett, P. D. *J. Am. Chem. Soc.* **1966**, *88*, 137–143.
(30) Howard, J. A.; Ingold, K. U. *Can. J. Chem.* **1966**, *44*, 1119–1130.
(31) Kiefer, H.; Traylor, T. G. *J. Am. Chem. Soc.* **1967**, *89*, 6667–6671.
(32) Neuman, R. C., Jr.; Bussey, R. J. *J. Am. Chem. Soc.* **1970**, *92*, 2440–2445.

Table III. Autoxidation of Egg Lecithin in Chlorobenzene at 30 °C under 760 torr of O_2 Initiated with Di-*tert*-butyl Hyponitrite

run no.	$10^3 \times$ [egg l]/ M	$10^3 \times$ [DBHN]/ M	$10^5 \times$ [α-Toc]/ M	$10^4\tau$/s	$10^8 \times (2k_i[\text{DBHN}])^a$/ M s^{-1}	$10^8 R_i$/ M s^{-1}	e	$-10^7 d[O_2]/dt$/M s^{-1} measd	corrd[b]	ν^c	$-d[O_2]/dt/[\text{egg l}]R_i^{1/2}$[c]/ M$^{-1/2}$ s$^{-1/2}$
7[d]	2.72	3.18			2.04	1.35[e]		2.23	2.27	17	0.72
8[d]	10.6	3.30			2.11	1.39[e]		7.45	7.49	54	0.60
9[d]	2.72	3.18			2.04	1.35[e]		2.13	2.17	16	0.69
10[d]	2.72	3.18			2.04	1.35[e]		2.52	2.56	19	0.81
11[d]	2.72	3.18			2.04	1.35[e]		1.84	1.88	14	0.60
11a[d]	10.9	2.98			1.91	1.26[e]		7.12	7.15	57	0.59
11b[d]	9.8	15.3			9.79	6.46[e]		18.4	18.5	29	0.74
33[f]	2.56	0.75	1.02	6.50	0.48	0.31	0.65	0.67	0.68	22	0.47
34[f]	10.0	2.93	5.00	7.48	1.88	1.34	0.71	7.00	7.03	53	0.61
35[f]	10.0	2.93	1.00	1.45	1.88	1.38	0.73	6.95	6.98	51	0.59
36[f]	2.50	2.93	2.50	3.66	1.88	1.37	0.73	1.46	1.49	11	0.51
37a[f]	2.50	2.93	2.50	3.87	1.88	1.29	0.69	1.73	1.76	14	0.62
37b[f]	7.18	2.80	2.39	4.01	1.79	1.19	0.67	4.39	4.42	37	0.57
38a[f]	2.50	11.72	2.50	1.09	7.50	4.59	0.61	3.45	3.58	8	0.67
38b[f]	7.32	11.43	4.88	2.03	7.32	4.81	0.66	6.95	7.07	15	0.44
39[f]	12.5	2.93	0.40	0.79	1.88	1.01	0.54	6.95	6.98	69	0.55
40[f]	2.50	2.93	4.55	8.23	1.88	1.11	0.59	1.83	1.86	17	0.71
41a[f]	12.5	2.93	1.37	2.19	1.88	1.25	0.66	7.05	7.08	57	0.51
41b[f]	12.5	2.93			1.88	1.25	0.66	5.90[g]	5.93	47	(0.43)[h]

[a] Based on $k_i = 3.2 \times 10^{-6}$ s^{-1} (see text). [b] Same as footnote *a*, Table I. [c] Calculated from corrected rate. [d] Egg lecithin supplied by Anne Joyce. [e] Based on $e = 0.66$ obtained from runs 33–41. [f] Commercial egg lecithin. [g] In air, i.e., under ca. 159 torr of O_2. [h] Not included in calculations of average value for the oxidizability.

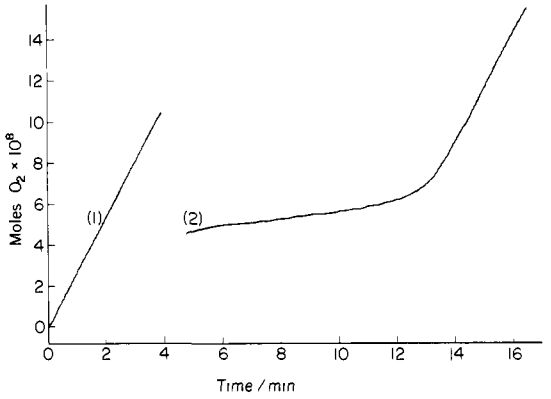


Figure 1. Autoxidation of egg lecithin (40 mg) in chlorobenzene (2 mL), initiated with DBHN (2.34×10^{-5} mol): (1) uninhibited reaction; (2) α-tocopherol (3.6×10^{-8} mol) added after 4 min.

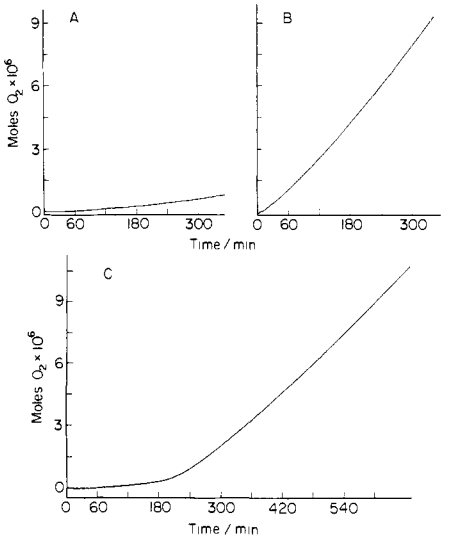


Figure 2. Autoxidation of egg lecithin (40 mg) in water (2 mL of 0.1 M NaCl): A, self-initiated reaction; B, initiated with DBHN (2.0×10^{-5} mol); C, a repeat of B, inhibited with α-tocopherol (5.0×10^{-8} mol).

aqueous dispersions are not sufficiently precise to conclude that there is definitely an effect on k_i due to the bilayer For all further calculations we use the Kiefer and Traylor[24] value of 3.2×10^{-6} s^{-1} for k_i at 30 °C since this is approximately the average of our own values. Any errors that arise in this way are small and do not affect our overall conclusions.

A typical trace for the autoxidation of egg lecithin in chlorobenzene initiated with DBHN and showing both the uninhibited and α-tocopherol-inhibited stages is illustrated in Figure 1. The kinetic data for egg lecithin from two sources with DBHN as initiator are summarized in Table III. In this system the efficiency of initiation by DBHN is 66 ± 6%. Corrections to the measured rate are small, though the chain lengths are relatively short because of the low concentrations of egg lecithin employed. The essential constancy of the numbers in the final column indicates that the autoxidation of egg lecithin in chlorobenzene obeys eq 5. The mean value for the oxidizability of egg lecithin at 30 °C initiated with DBHN and dissolved in chlorobenzene is 0.61 ± 0.09 M$^{-1/2}$. s$^{-1/2}$. The same value was obtained with other initiators.

The autoxidation of egg lecithin in 0.1 M aqueous sodium chloride exhibited characteristics which placed severe constraints on the experimental procedures. The autoxidation of egg lecithin is a self-initiated process (see Figure 2a). This causes no experimental problems in chlorobenzene solutions because the self-initiation process can be rendered relatively unimportant by the addition of a sufficient quantity of any of the usual thermal initiators. However, most initiators are almost completely ineffective at initiating the autoxidation of egg lecithin in an aqueous dispersion. That is, the "initiated" and the "uninitiated" oxidation traces are essentially identical, making a kinetic study impossible. This problem has two causes. In the first place, many common initiators are virtually insoluble in egg lecithin. This was not immediately apparent because the initiators were added to a homogeneous solution of egg lecithin in a volatile organic solvent which was later removed (see Experimental Section) with the nonvolatile residue then being dispersed in water by using a vortex stirrer. A milky looking liquid is formed. When it was discovered that AIBN, AC, and tetraphenylbutane were ineffective initiators, these dispersions were examined by phase contrast microscopy. In all three cases the initiator had crystallized out of the dispersion—the crystals being filtered and identified by their melting points. Second, the initiation efficiency of an initiator which is soluble in egg lecithin is extremely low (vide infra). The *only* satisfactory initiator we could discover for egg lecithin aqueous dispersions which would "swamp out" the self-initiation process was DBHN and so all "useful" experiments were done with this compound.

Table IV. Determination of R_i and e for the Di-*tert*-butyl Hyponitrite Initiated, α-Tocopherol-Inhibited Autoxidation of Egg Lecithin in 0.1 M Aqueous Sodium Chloride at 30 °C under 760 torr of O_2

run no.	10^6[egg l][a]/ mol	[DBHN][b]/ mol L^{-1}	10^2[α-Toc][b]/ mol L^{-1}	$10^4\tau$/s	$10^7(2k_i$[DBHN])[b,c]/ mol L^{-1} s^{-1}	10^7R_i[b]/ mol L^{-1} s^{-1}	e[d]
24	25	0.80	0.20	1.63	51.2	2.45	0.048
65	25	0.80	0.20	2.43	51.2	1.65	0.032
66	25	0.80	0.10	1.33	51.2	1.50	0.029
67	50	0.40	0.10	1.38	25.6	1.45	0.057
68	50	0.40	0.20	4.11	25.6	0.97	0.038
69	50	0.40	0.20	3.29	25.6	1.22	0.048
70	25	0.20	0.20	7.41	12.8	0.54	0.042
71	25	0.20	0.10	4.51	12.8	0.44	0.034
72	25	0.20	0.04	1.41	12.8	0.57	0.045
73	25	0.20	0.08	3.04	12.8	0.53	0.041
74	5	0.80	0.20	1.41	51.2	2.80	0.055

[a] Same as footnote *a*, Table II. [b] Same as footnote *c*, Table II. [c] Based on $k_i = 3.2 \times 10^{-6}$ s^{-1} (see text). [d] See Note Added in Proof.

Table V. Autoxidation of Egg Lecithin in 0.1 M Aqueous Sodium Chloride at 30 °C under 760 torr of O_2 Initiated with Di-*tert*-butyl Hyponitrite

run no.	10^6[egg l][a]/ mol	[DBHN][b]/ mol L^{-1}	-10^6d[O_2]/dt[b]/mol L^{-1} s^{-1} measd[c]	corrd[d]	ν[e,f]	-10^2d[O_2]/dt/ $R_i^{1/2}$[e,f]/ $mol^{-1/2}$ $L^{1/2}$ $s^{-1/2}$
19	25	0.80	5.84	8.30	38	1.78
25	5	0.80	4.40	6.86	32	1.47
26	25	0.80	5.84	8.30	38	1.78
27	25	0.80	5.68	8.14	37	1.74
28	5	0.80	6.80	9.26	43	1.98
29	5	0.80	4.60	7.06	32	1.51
53	50	0.10	1.78	2.08	76	1.26
57	25	0.20	4.08	4.70	86	2.01
58	25	0.20	3.40	4.02	74	1.72
61	25	0.20	2.52	3.14	58	1.34
63	25	0.80	6.60	9.06	42	1.94
76	50	0.10	1.82	2.12	78	1.29
77	50	0.10	2.34	2.64	97	1.60
78	25	0.80	3.71[g]	6.17	28	(1.32)[h]
79	25	0.80	3.24[g]	5.70	26	(1.22)[h]

[a] Same as footnote *a*, Table II. [b] Same as footnote *c*, Table II. [c] Rates for 5, 25, and 50 × 10^{-6} mol of egg lecithin were measured from slopes of oxygen absorption traces to 1.5 × 10^{-7}, 7.5 × 10^{-7}, and 1.5 × 10^{-6} mol of O_2, respectively, so that percentage consumption of egg lecithin (3%) were equal. [d] Same as footnote *a*, Table I. [e] Same as footnote *b*, Table I. [f] Based on an R_i calculated by taking $e = 0.043$. See Note Added in Proof. [g] In air, i.e., under ca. 159 torr of O_2. [h] Not included in calculation of average value for the oxidizability.

As mentioned above, the egg lecithin dispersions were prepared by vortex stirring. This yielded vesicles of various sizes. The majority had a diameter of ca. 2–6 μm, which means that they were of a size similar to that of many types of cells.[33] There were also some larger aggregates. Ultrasonic irradiation is used in many different types of experiments with vesicles to produce very much smaller, but uniformly sized, unilamellar vesicles.[17,34-39] This seemed not only unnecessary but also probably counterproductive for experiments designed to study the autoxidation behavior of model cell membranes. For one thing, if the volume of each particle of substrate is made sufficiently small, the two radicals formed by the thermal decomposition of an initiator will be unable to diffuse completely away from one another. The radicals will therefore react with one another rather than initiating independent autoxidation chains. In addition, prolonged sonication for 1 h or more is generally required to produce uniform, unilamellar vesicles, even with special high-powered cup sonicators.[39] Such treatment would tend to destroy thermal initiators to judge from the fact that relatively mild sonication (2 h at 10 °C in a bath sonicator) caused appreciable decomposition of DBHN.

The inhibitor, α-tocopherol, had to be added in the same way as the initiator because we were unsuccessful in our attempts to insert it into the egg lecithin during an autoxidation. The techniques tried included the addition of α-tocopherol as a solution in acetonitrile, as a film on a glass surface, and in separately prepared dispersions in egg lecithin or sodium dodecyl sulfate. In no case was the autoxidation inhibited.

Because the α-tocopherol was added at the start of a run, the rates of chain initiation and hence the efficiency of initiation were measured independently of the experiments in which the initiated rate of oxidation was determined. A typical inhibition trace is shown in Figure 2c. The results are summarized in Table IV. In calculating the efficiency of initiation by DBHN in the aqueous egg lecithin dispersions, we have taken k_i to be 3.2 × 10^{-6} s^{-1} (vide supra). It has also been assumed that the α-tocopherol resides wholly in the egg lecithin and that it traps two peroxyls (just as in the homogeneous solution). The first assumption is supported by the very low solubility of α-tocopherol in water (≤1.0 × 10^{-7} M at 22 °C). The second assumption cannot be independently verified, but we see no reason to doubt its validity.[40] On this basis, the efficiency of initiation was calculated to be 9.1 ± 0.9% (see Note Added in Proof), which is so much smaller than the efficiency (66%) found for egg lecithin in chlorobenzene that we also explored the possibility that the DBHN resided mainly in the aqueous

(33) Metzler, D. E. "Biochemistry"; Academic Press: New York, 1977; p 13.
(34) Attwood, D.; Saunders, L. *Biochim. Biophys. Acta* **1966**, *116*, 108–113.
(35) Johnson, S. M.; Bangham, A. D.; Hill, M. W.; Korn, E. D. *Biochim. Biophys. Acta* **1971**, *233*, 820–826.
(36) Finer, E. G.; Flook, A. G.; Hauser, H. *Biochim. Biophys. Acta* **1972**, *260*, 49–58.
(37) Papahadjopoulos, D.; Nir, S.; Ohki, S. *Biochim. Biophys. Acta* **1972**, *266*, 561–583.
(38) Huang, C.; Thompson, T. E. *Methods Enzymol.* **1974**, *32*, 485–489.
(39) Barrow, D. A.; Lentz, B. R. *Biochim. Biophys. Acta* **1980**, *597*, 92–99.

(40) The α-tocopherol *must* trap at least one peroxyl per molecule. If this were all it trapped, the efficiency of initiation would rise to 8.6 ± 1.6% but none of our conclusions would require major change.

Table VI. Major Volatile Organic Products Formed from Di-*tert*-butyl Hyponitrite after 25 Days at 30 °C

	$10^6 \times [DBHN]_0$/ mol	$10^6 \times [Me_3COOCMe_3]$/ mol	$10^6 \times [Me_3COH]$/ mol	$10^6 \times [Me_2CO]$/ mol	% $Me_3CO\cdot$ recovered
20 mg of egg l in 2 mL of chlorobenzene[a] under 760 torr of O_2	33.6	5.92	29.4	0	61.4
20 mg of egg l in 2 mL of 0.1 M aqueous NaCl[b,c] under 760 torr of O_2	20.0	3.24 (3.24:0)	1.91 (1.05:0.86)	1.62 (1.32:0.30)	25.0[e]
20 mg of egg l in 2 mL of 0.1 M aqueous NaCl[b,d] under 760 torr of N_2	20.0	4.60 (4.60:0)	2.49 (2.29:0.20)	2.10 (1.74:0.36)	34.5[e]

[a] Internal standard was 2-propanol. [b] Internal standard was trichloroethylene. Some of the organic phase was dispersed into the aqueous phase even after centrifugation. This required a correction to the product yields in the aqueous phase which was based on the amount of C_2HCl_3 found in this phase. The numbers in brackets beside the total yields are the corrected yields in the organic and aqueous phases, respectively. [c] Aqueous phase contained 16% organics. [d] Aqueous phase contained 5.6% organics. [e] See Note Added in Proof.

Table VII. Analysis of Egg Lecithin Fatty Acids

	acids (as methyl esters)[a]								
egg lecithin sample	12:0	14:0	16:0	16:1	18:0	18:1	18:2	20:4	% unsaturation
unoxidized	0.23	0.12	35.4	1.9	11.8	29.0	19.0	2.6	52.5
oxid in C_6H_5Cl	0.48	0.48	55.2	2.1	16.2	25.0	0.59	0	27.7
oxid in 0.1 M aqueous NaCl	0.28	0.18	40.9	2.1	12.4	30.7	13.5	0	46.3

[a] Each fatty acid ester is given as a percentage of the total. For each of the column headings the first number refers to the number of carbon atoms in the fatty acid, while the second refers to the number of double bonds.

phase. However, UV analysis of the clear aqueous filtrate from an ultrafiltration showed that under typical experimental conditions less than 0.1% of the DBHN had partitioned into the aqueous phase. This small quantity[41] was consistent with the result of a separate experiment which gave the solubility of DBHN in water as 1.3×10^{-5} M at 22 °C. We conclude, therefore, that the small magnitude of *e* for DBHN in the egg lecithin dispersions is a genuine phenomenon and is not an experimental artifact.

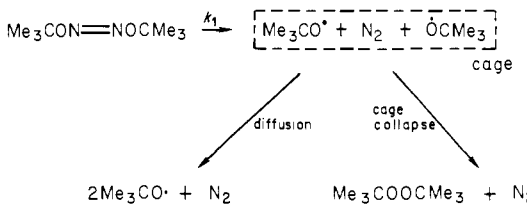
The initial rates of the DBHN initiated, uninhibited, autoxidation of egg lecithin in aqueous dispersion are summarized in Table V and a typical trace is shown in Figure 2b. In calculating reagent concentrations, etc., for this table (as for Table IV, also) we had to make an allowance for the fact that the egg lecithin is *not* diluted by the water. That is, *the egg lecithin concentration in a bilayer is the same as in the bulk material.* For purposes of calculation we assumed that the average molecular weight of egg lecithin was 800 and that its density was 0.8. In this way the volume of egg lecithin used is defined as being equal to the number of moles; i.e., the egg lecithin concentration is unity in all runs. In these autoxidations the corrections to the measured rates are quite large because of the low efficiency of chain initiation. Chain lengths are substantial. The near constancy of the numbers in the final column indicates that the autoxidation of egg lecithin in aqueous dispersion follows classical kinetics, i.e., obeys eq 5. However, the mean value for the oxidizability of egg lecithin in 0.1 M aqueous sodium chloride at 30 °C is only $(1.65 \pm 0.25) \times 10^{-2}$ $M^{-1/2}$ $s^{-1/2}$, which is very much smaller than the value found in homogeneous solution in chlorobenzene, viz. 0.61 $M^{-1/2}$ $s^{-1/2}$.

The yields of the three major organic products from DBHN formed during egg lecithin autoxidations in chlorobenzene and water are given in Table VI. The fatty acid composition for fresh and for autoxidized egg lecithin are given in Table VII.

Discussion

Each of the three questions posed in the beginning will be dealt with in turn.

(1) The Question of Initiator Efficiency. The efficiency of chain initiation by DBHN, as measured by the induction period method, is a respectable 66% in chlorobenzene (Table III) but is only 9.1% in the egg lecithin bilayer in 0.1 M aqueous NaCl (see Note Added in Proof). These efficiencies can be independently checked, in principle, by analysis of the main organic products formed from the DBHN.[31] The relevant reactions are shown in eq 10–12. Only



(10)

$$Me_3CO\cdot + RH \rightarrow Me_3COH + R\cdot \quad (11)$$

$$Me_3CO\cdot \rightarrow Me_2CO + Me\cdot \quad (12)$$

that fraction of *tert*-butoxyls that diffuse out of the solvent cage can initiate autoxidation chains. Initiation will occur primarily by reaction 11. The β-scission process (reaction 12) does not occur to a significant extent in chlorobenzene. Some acetone is, however, formed in the aqueous dispersion (see Table VI) which suggests that β-scission involves only those *tert*-butoxyls which are formed in, or diffuse into, the aqueous phase. This can be readily understood because it is well-known that polar solvents,[42] and more particularly, hydrogen-bonding solvents,[43] greatly accelerate reaction 12. We believe that methyl and its product methylperoxyl, formed in the aqueous phase, are unlikely to diffuse back into the bilayer and initiate autoxidation. On this basis, the efficiency of initiation after complete destruction of the DBHN is probably best represented by eq 13.

$$e = [Me_3COH]/2[DBHN]_0 \quad (13)$$

The analytical results under 760 torr of O_2 in chlorobenzene and in 0.1 M aqueous sodium chloride (see Table VI) yield *e* values of 0.44 and 0.048, respectively, according to this equation. Unfortunately, the material balances are so poor that although these results are consistent with the efficiencies determined by the induction period method (i.e., 0.66 and 0.091), they hardly constitute supporting evidence for these values (however, see Note Added in Proof). We suggest that the poor material balances are due to the fact that autoxidation of the methylene interrupted diene systems of polyunsaturated fatty acids produces conjugated dienes and trienes. *tert*-Butoxyl radicals *add* to conjugated dienes, rather than abstract hydrogen from them.[44,45] The *tert*-butoxyls are

(41) Reviewer I suggested that ultrafiltration may have led to some absorption of DBHN on the filter. While this may have occurred, ultrafiltration was not employed in the direct measurement of DBHN's solubility in water. There can be no question regarding the hydrophobicity of DBHN.

(42) Walling, C.; Padwa, A. *J. Am. Chem. Soc.* **1963**, *85*, 1593–1597.
(43) Walling, C.; Wagner, P. *J. Am. Chem. Soc.* **1963**, *85*, 2333–2334.
(44) Elson, I. H.; Mao, S. W.; Kochi, J. K. *J. Am. Chem. Soc.* **1975**, 97, 335–341.
(45) Davies, A. G.; Griller, D.; Ingold, K. U.; Lindsay, D. A.; Walton, J. C. *J. Chem. Soc., Perkin Trans. 2*, **1981**, 633–41.

therefore trapped by the egg lecithin and do not form volatile products.[46]

It has been shown[31,32] that the efficiency of initiation by DBHN (and related thermal initiators) decreases as the viscosity of the medium increases, though the change in the cage effect with solvent does not appear to follow any simple function of the viscosity. Lipid bilayers in water have been shown by a variety of techniques to have relatively high microviscosities (e.g., relatively long rotational correlation times) in the polar, head group, region and somewhat lower microviscosities (e.g., relatively shorter rotational correlation times) well inside the hydrophobic region.[47-49] There are inherent difficulties in the concept and measurement of microviscosities because different results are obtained with different "probe" molecules.[49] In fact, the relevance of most microviscosity measurements as regards the ease of translational motion of a small molecule or radical is open to question. Nevertheless, there can be no doubt that the average viscosity of the hydrophobic region of a phospholipid bilayer is considerably greater than the viscosity of a mixture of fatty acid esters. Thus, for egg lecithin bilayers the following microviscosities at 30 °C can be calculated from data obtained by using various hydrophobic "probes": 58 and 73 cP;[50] 82 cP;[51] and 96 cP.[52] The very much lower efficiency of initiation by DBHN in the egg lecithin aqueous dispersion compared with its value in chlorobenzene we attribute to thhe overall higher microviscosity of the bilayer. The values of e found in this work for DBHN are compared in Table VIII with some of Kiefer and Traylor's data[31] for this initiator.

Our results with DBHN contrast with those of Winterle and Mill[20] who used ABCP as their initiator (see introduction). At 50 °C in phospholipid bilayers e values for ABCP were ca. 0.21–0.25 compared with ca. 0.45 in organic compounds having a viscosity of about 1 cP. Dr. Mill has suggested that the difference in initiation efficiencies of DBHN and ABCP is due to the fact that the DBHN locates itself near the viscous head groups, hence the low e, whereas the ABCP locates itself more deeply within the bilayer in a region of lower viscosity (estimated to be only about 8 cP[20]), hence the larger e. An independent determination of the location of DBHN and ABCP within a phospholipid bilayer would be most revealing.

(2) The Question of the Kinetic Rate Law. Our results provide the first unequivocal evidence that the autoxidation under 760 torr of O_2 of egg lecithin in a homogeneous solution (Table III) and as an organized lipid bilayer in an aqueous dispersion (Table V) follow the same rate law and that this law is the classical kinetic law for hydrocarbon autoxidation, (eq 5). There is probably a small dependence of the rate on the oxygen partial pressure when this is reduced from 760 to 159 torr (see run 41b of Table III and runs 78 and 79 of Table V). However, this small reduction in rate is quite normal for substrates containing methylene-interrupted diene units.[53] The substituted pentadienyl radicals that are formed are so strongly resonance stabilized that the addition of oxygen (reaction 2) is significantly reversible under normal autoxidation conditions.[21,54] At reduced oxygen pressures chain termination by the cross reaction

$$R\cdot + ROO\cdot \rightarrow \text{nonradical products} \qquad (14)$$

Table VIII. Effect of the Viscosity of the Medium on DBHN Initiation Efficiency

medium	T/°C	viscosity/ cP	e	ref
chlorobenzene	30	0.73	0.66	this work
Tetralin	30	1.83	0.57	this work
n-octadecane	45	2.77	0.35	31
100% Nujol	45	79.9	0.16	31
egg l in H_2O	30	58–96[a]	0.091	this work

[a] See ref 50–52.

can be important. That substituted pentadienyls are formed is indicated by the fact that the linoleate (18:2) and arachidonate (20:4) esters are the most readily autoxidized fractions in the egg lecithin (see Table VII).

(3) The Question of Oxidizability. The oxidizability of egg lecithin at 30 °C in vesicles is only 2.7% of that for the homogeneous material. That is, $k_p/(2k_t)^{1/2}$ has a value of 0.0165 $M^{-1/2}$ $s^{-1/2}$ in the aqueous dispersion and 0.61 $M^{-1/2}$ $s^{-1/2}$ in chlorobenzene. We are unaware of any published work that might have led us to anticipate this result.

To attempt, in a preliminary way, to explain this result, we start by comparing the oxidizability of egg lecithin with that of structurally related model compounds and we consider the actual k_p and $2k_t$ values reported for these models. In chlorobenzene at 30 °C, methyl linoleate has $k_p/(2k_t)^{1/2}$ = 0.021 $M^{-1/2}$ $s^{-1/2}$, k_p = 62 M^{-1} s^{-1}, and $2k_t$ = 8.8 × 10^6 M^{-1} s^{-1}, while methyl linolenate has $k_p/(2k_t)^{1/2}$ = 0.039 $M^{-1/2}$ $s^{-1/2}$, k_p = 236 M^{-1} s^{-1}, and $2k_t$ = 3.6 × 10^7 M^{-1} s^{-1}.[55] It is immediately obvious that egg lecithin in chlorobenzene is, relatively, highly oxidizable. It is extremely unlikely that this is due to any appreciable decrease in $2k_t$,[10,11,55-58] and this means that k_p must be quite large (perhaps ca. 3 × 10^3 M^{-1} s^{-1}) for egg lecithin dissolved in chlorobenzene.

The reduced oxidizability of egg lecithin in the aqueous dispersion cannot be due to a *simple* increase in $2k_t$. This is because a reaction with a rate constant of ca. 10^7 M^{-1} s^{-1} (cf. $2k_t$ for methyl linoleate and linolenate) would be proceeding at close to the diffusion-controlled rate in a medium having the high viscosity of egg lecithin vesicles (ca. 80 cP, vide supra). There must, therefore, be a dramatic reduction of k_p in the aqueous dispersion. Why? We suggest that the answer lies in the polarity of the peroxyl radical which has not hitherto been explicitly recognized.

Nitroxide radicals, $R_2NO\cdot$, which are isoelectronic with peroxyls, have a large dipole moment (ca. 3 D)[59] which can be attributed to extensive (ca. 50%) conjugative delocalization of the unpaired electron onto the nitrogen.[60] The same phenomenon will occur in peroxyls since the unpaired electron has been shown to be ca. 60% on the terminal oxygen and 40% on the inner oxygen.[61-64] That is, canonical structure **2**, which contributes to the stabilization of peroxyls, induces a dipole moment.

$$R-\ddot{O}-\dot{O} \longleftrightarrow R-\ddot{O}^{+}-\ddot{O}^{-}$$

1 (60%) 2 (40%)

The magnitude of the peroxyl dipole moment can be estimated from the spin density on the inner oxygen (which equals the charge on each oxygen) and the O–O bond length. The latter should lie between the ca. 1.47 Å typical of the O–O bond in peroxides[65]

(46) This problem is more pronounced under autoxidation conditions because conjugated dienes and trienes will be formed in higher yields. Thus, more *tert*-butoxyls could be accounted for in the experiment with an aqueous dispersion of egg lecithin that was run under nitrogen than for the run under oxygen (see Table VI).

(47) Hubbell, W. L.; McConnell, H. M. *J. Am. Chem. Soc.* **1971**, 93, 314–326.
(48) Levine, Y. K.; Birdsall, N. J. M.; Lee, A. G.; Metcaffe, J. C. *Biochemistry* **1972**, *11*, 1416–1421.
(49) Seelig, J.; Seelig, A. *Q. Rev. Biophys.* **1980**, *13*, 19–61.
(50) Shinitzky, M.; Barenholz, Y. *J. Biol. Chem.* **1974**, *249*, 2652–2657.
(51) Hare, F.; Amiell, J.; Lussan, C. *Biochim. Biophys. Acta* **1979**, *555*, 388–408.
(52) Cogan, U.; Shinitzky, M.; Weber, G.; Nishida, T. *Biochemistry* **1973**, *12*, 521–528.
(53) Bateman, L. *Q. Rev., Chem. Soc.* **1954**, *8*, 147–167.
(54) Chan, H. W. S.; Levett, G.; Matthew, J. A. *Chem. Phys. Lipids* **1979**, *24*, 245–256.
(55) Howard, J. A.; Ingold, K. U. *Can. J. Chem.* **1966**, *45*, 793–802.
(56) Ingold, K. U. *Pure Appl. Chem.* **1967**, *15*, 49-67.
(57) Ingold, K. U. *Proc. World Pet. Congr.* **1967**, *7*, 15–22.
(58) Howard, J. A.; Ingold, K. U. *Can. J. Chem.* **1968**, *46*, 2661–2668.
(59) Rozantsev, E. G.; Gur'yanova, E. M. *Izv. Adad. Nauk SSSR, Ser. Khim.* **1966**, 979–983.
(60) Mendenhall, G. D.; Ingold, K. U. *J. Am. Chem. Soc.* **1973**, *95*, 6390–6394.
(61) Adamic, K.; Ingold, K. U.; Morton, J. R. *J. Am. Chem. Soc.* **1970**, *92*, 922–923.
(62) Howard, J. A. *Can. J. Chem.* **1972**, *50*, 1981–1983.
(63) Howard, J. A. *Can. J. Chem.* **1979**, *57*, 253–254.
(64) Melamud, E.; Silver, B. L. *J. Phys. Chem.* **1973**, *77*, 1896–1900.
(65) Silbert, L. S. In "Organic Peroxides"; Swern, D., Ed.; Wiley-Interscience: New York, 1971; Vol. II, Chapter 7, pp 637–798.

and the ca. 1.28 Å typical of the N–O bond length in nitroxides.[66] Taking a value of 1.35 Å, the dipole moment of peroxyl is given by eq 15.

$$\mu = (0.4 \times 4.8 \times 10^{-10} \text{ esu}) \times 1.35 \times 10^{-8} \text{ cm} = 2.6 \text{ D}$$

The physical consequence of peroxyl polarity will be that immediately after its formation deep within the lipid bilayers the peroxyl portion of the molecule will diffuse rapidly away from the nonplar hydrocarbon environment in which it was formed and into the polar surface region of the bilayer.[67] In this surface region there will be little or no material that is readily attacked by a peroxyl, i.e., that is readily autoxidizable. Chain propagation will therefore be retarded (i.e., k_p will decrease). Furthermore, the *local* concentration of peroxyls in the surface region will be greater than their average concentration if they were uniformly distributed throughout the entire volume of the bilayer. With an increased local concentration of peroxyls the rate of peroxy–peroxyl reactions will be increased; i.e., in effect $2k_t$ will be increased.

The foregoing interpretation, *although of necessity somewhat speculative*, should provide a useful model for further studies.

Two additional points are, we believe, worth making. First, our conclusion that k_p has a lower value in the bilayer is at first sight inconsistent with Porter el al.'s[21] observation that k_p/k_{-2} has about the same value in a bilayer as a homogeneous solution (see introduction). Our two observations could be reconciled if changes in k_{-2} parallel the changes in k_p.[68] That is, the rate constant for the β-scission of a peroxyl radical would have to be smaller in polar than in nonpolar media. There is, unfortunately, no experimental evidence regarding this matter. Second, it has been suggested by Dr. Ted Mill that the low oxidizability we observe with the aqueous dispersions of egg lecithin could be due to the fact that the DBHN may be located and hence initiate chains, in the head group region of the bilayer where oxidizability of the adjacent fatty acid chains is low (because they are paraffinic, i.e., contain only saturated C–H bonds). The difference between this view and our own proposal that any peroxyl formed within the bilayer will tend to locate itself in (i.e., float into) the polar region is quite subtle. It would be difficult to distinguish experimentally between the two possibilities, particularly since both effects may play a role in reducing the oxidizability of phospholipid bilayers initiated with DBHN. At the present time we rather prefer our own suggestion (that autoxidation involves the reactive portion of the bilayer and that the peroxyls tend to be expelled from this portion) for the following reasons. (i) The location of the DBHN in the bilayer is unknown. In view of its hydrophobicity, steric crowding of the heteroatoms, and low polarity, we expect it to be located well within the bilayer and not in the head group region. (ii) The polyunsaturated fatty acids in the bilayer certainly are autoxidized preferentially (see Table VII). (iii) Peroxyl radicals must have a strong dipole moment so they certainly should prefer to locate themselves in regions of high polarity. It should be noted that this physical relocation (diffusion to the surface of the bilayer) should be easily competitive with hydrogen atom abstraction within the bilayer since even for polyunsaturated fatty acids k_p is not particularly large (ca. 100 M^{-1} s^{-1}/doubly allylic CH_2 group[55]).

In conclusion, our results suggest that both chemical and physical factors retard the rates of autoxidation of membranes in biological systems. The prime chemical effect is the use in many systems, including man, of Vitamin E as a chain-breaking antioxidant. As we have shown,[26] α-tocopherol has the optimum phenolic moiety for rapid reaction with peroxyls. In addition, the overall structure of Vitamin E ensures that it is concentrated in the region in which it must work, i.e., in the lipid membranes. A physical effect may also be operative if, as seems likely, the phenolic head groups of tocopherols are oriented toward, and reside mainly within, the polar surface layers of the lipid bilayer since it is in this region that we believe the peroxyl radicals are themselves concentrated. Other physical effects which operate to retard the rates of autoxidation of membranes are the inefficiency of chain initiation which arises from the high microviscosity of the bilayer, and the "expulsion" of peroxyls from the nonpolar, readily autoxidized region of the membrane into the polar, inert region with a consequent decrease in the propagation rate and increase in the bimolecular peroxyl–peroxyl termination rate. No doubt other chemical and physical effects will be discovered which also operate in a manner which tends to retard the autoxidation of bilayers and biomembranes.

Note Added in Proof: In current work we analyze the aqueous dispersions of egg lecithin containing initiator to determine the DBHN content, using the HPLC method (see Experimental Section). We have found that removing the solvent (methylene chloride) in the cold with a stream of nitrogen followed by evacuation *causes a loss of up to one-half of the DBHN*. This affects our original calculations of initiator efficiency ($e = 0.043 \pm 0.008$, see Table IV), since the value calculated for $2k_t$[DBHN] involved the assumption that all of the initiator remained in the sample. A series of new experiments were carried out to measure R_i and e by the inhibitor method as described *but* with the samples also being analyzed for DBHN immediately before the run was started. The new value for e determined in this way was 0.091 ± 0.009. These new analytical results also imply that the material balances of the products from the decomposition of DBHN in the bilayers are not nearly as poor as we initially estimated (see Table VI and text). Our new results do not, however, alter our findings regarding the oxidizability of egg lecithin bilayers. In order to confirm this, the oxidizabilities were measured on dispersions of egg lecithin from three different sources which contained analyzed amounts of DBHN. The averaged oxidizability found, $(1.53 \pm 0.15) \times 10^{-2}$ $M^{-1/2}$ $s^{-1/2}$, is the same, within experimental error, as the values given in Table V which led to an averaged value of $(1.65 \pm 0.25) \times 10^{-2}$ $M^{-1/2}$ $s^{-1/2}$. We are indebted to Miss J. VanKessel, Mount Allison University, for these recent results.

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(66) (a) Lajzêrowicz-Bonneteau, J. *Acta Crystallogr., Sect. B* **1968**, *B24*, 196–199. (b) Boeyens, J. C. A.; Kruger, G. J. *Ibid* **1970**, *B26*, 668–672. (c) Capiomont, A.; Chion, B.; Lajzêrowicz, J. *Ibid.* **1971**, *B27*, 322–326. (d) Capiomont, A. *Ibid.* **1972**, *B28*, 2298–2301. (e) Chion, B.; Capiomont, A.; Lajzêrowicz, J. *Ibid.* **1972**, *B28*, 618–619. (f) Turley, J. W.; Boer, F. P. *Ibid.* **1972**, *B28*, 1641–1644. (g) Capiomont, A. *Ibid.* **1973**, *B29*, 1720–1722. (h) Capiomont, A.; Lajzêrowicz-Bonneteau, J. *Ibid.* **1974**, *B30*, 2160–2166. (i) Chion, B.; Thomas, M. *Ibid.* **1975**, *B31*, 472–473. (j) Chion, B.; Lajzêrowicz, J. *Ibid.* **1975**, *B31*, 1430–1435.

(67) Reviewer 1 pointed out that nitroxyl spin labels are not extruded from bilayers. We regard this fact, of which we were well aware, as quite irrelevent because, in all such experiments done to date, the NO group has been buried within its own, personalized, hydrocarbon environment from which it cannot escape. That is, typical spin probes have two tertiary carbon atoms attached to the nitrogen and the NO function is completely enveloped by the groups attached to these carbons. We predict that a sterically unhindered nitroxyl spin probe placed in a phospholipid bilayer would indeed locate itself in the polar region.

(68) Suggested by reviewer II.